Biochem. J. (1988) 252, 207–213 (Printed in Great Britain)

25-Hydroxylation of vitamin D_3 by a cytochrome *P*-450 from rabbit liver mitochondria

Helena DAHLBÄCK and Kjell WIKVALL
Department of Pharmaceutical Biochemistry, University of Uppsala, Box 578, S-751 23 Uppsala, Sweden

A cytochrome *P*-450 catalysing 25-hydroxylation of vitamin D_3 was purified from liver mitochondria of untreated rabbits. The enzyme fraction contained 9 nmol of cytochrome *P*-450/mg of protein and showed only one protein band with an apparent M_r of 52000 upon SDS/polyacrylamide-gel electrophoresis. The preparation showed a single protein spot with an apparent isoelectric point of 7.8 and an M_r of approx. 52000 upon two-dimensional isoelectric-focusing–polyacrylamide-gel electrophoresis. The purified cytochrome *P*-450 catalysed 25-hydroxylation of vitamin D_3 up to 5000 times more efficiently than did the mitochondria. The cytochrome *P*-450 required both ferredoxin and ferredoxin reductase for catalytic activity. Microsomal NADPH–cytochrome *P*-450 reductase could not replace ferredoxin and ferredoxin reductase. The cytochrome *P*-450 catalysed, in addition to 25-hydroxylation of vitamin D_3, the 25-hydroxylation of 1α-hydroxyvitamin D_3 and the 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol. The enzyme did not catalyse side-chain cleavage of cholesterol, 11β-hydroxylation of deoxycorticosterone, 1α-hydroxylation of 25-hydroxyvitamin D_3, hydroxylations of lauric acid and testosterone or demethylation of benzphetamine. The results raise the possibility that the 25-hydroxylation of vitamin D_3 and the 26-hydroxylation of C_{27} steroids are catalysed by the same species of cytochrome *P*-450 in liver mitochondria. The possible role of the liver mitochondrial cytochrome *P*-450 in the metabolism of vitamin D_3 is discussed.

INTRODUCTION

The first step in the bioactivation of vitamin D_3 is a 25-hydroxylation. The reaction is catalysed by both the microsomal [1,2] and the mitochondrial [3] fractions of the liver. A microsomal cytochrome *P*-450 catalysing the 25-hydroxylation has been purified to apparent homogeneity from rat liver microsomal fraction [4–6]. The involvement of cytochrome *P*-450 in the mitochondrial 25-hydroxylation of vitamin D_3 was suggested by Björkhem & Holmberg [3] from studies with intact rat liver mitochondria. More direct evidence for the participation of cytochrome *P*-450 was provided by the reconstitution experiments performed by Pedersen, Björkhem and their co-workers [7–9]. These authors have shown 25-hydroxylase activity in reconstituted systems containing partially purified cytochrome *P*-450, ferredoxin and ferredoxin reductase from rat [7,8] and human [9] liver mitochondria. The 25-hydroxylase activity was, however, not higher in the reconstituted systems than in the mitochondria and the cytochrome *P*-450 fractions did not contain more than 0.2 nmol of cytochrome *P*-450/mg of protein. Recently we reported the isolation from rat liver mitochondria of a partially purified cytochrome *P*-450 fraction that catalysed 25-hydroxylation of vitamin D_3 more effectively than did intact mitochondria [10]. The cytochrome *P*-450 fraction was not pure and showed several protein bands upon gel electrophoresis. Thus it has not been possible to define the species of cytochrome *P*-450 involved in mitochondrial 25-hydroxylation of vitamin D_3.

The present paper reports the purification from rabbit liver mitochondria of an electrophoretically homogeneous cytochrome *P*-450 active in the 25-hydroxylation of vitamin D_3. Rabbit liver mitochondria were chosen in view of the fact that it is only from this source that electrophoretically homogeneous cytochrome *P*-450 active in the 26-hydroxylation of C_{27} steroids has been previously isolated [11].

EXPERIMENTAL

Materials

25-Hydroxy[23,24(n)-^{3}H]vitamin D_3 (107 Ci/mmol), [4-^{14}C]cholesterol (61 Ci/mol), [1-^{14}C]lauric acid (26 Ci/mol) and [4-^{14}C]testosterone (50 Ci/mol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Unlabelled 25-hydroxyvitamin D_3 and 1α-hydroxyvitamin D_3 were obtained respectively from Duphar, Weesp, The Netherlands, and Lövens, Malmö, Sweden. 5β-[7β-^{3}H]Cholestane-3α,7α,12α-triol (500 Ci/mol) was synthesized as described previously [12]. Benzphetamine hydrochloride was generously given by Dr. B. Lindeke (Department of Pharmaceutical Organic Chemistry, Uppsala, Sweden). Vitamin D_3, deoxycorticosterone, corticosterone, sodium cholate and cofactors were obtained from Sigma Chemical Co. Octylamine–Sepharose 4B was prepared as described previously [13]. Fractogel TSK DEAE-650(s) was obtained from Merck, Darmstadt, Germany, Emulgen 913 from Kao-Atlas, Tokyo, Japan, and hydroxyapatite (Bio-Gel HTP) from Bio-Rad Laboratories, Richmond, CA, U.S.A. Bio-Gel HTP was mixed with an equal amount of Whatman CF-1 cellulose powder before chromatography. The remaining chemicals were of reagent grade.

Enzyme purifications

Cytochrome *P*-450, catalysing 25-hydroxylation of vitamin D_3, was purified to electrophoretic homo-

geneity from liver mitochondria of untreated rabbits as described by Wikvall [11] with the modification that Fractogel TSK DEAE-650(s) was used instead of DE52 DEAE-cellulose.

Ferredoxin and ferredoxin reductase were prepared from bovine adrenal mitochondria as described previously [11].

Cytochrome *P*-450 was determined as described by Omura & Sato [14] and protein as described by Lowry *et al.* [15], with bovine serum albumin as standard. Ferredoxin and ferredoxin reductase concentrations were determined as described by Huang & Kimura [16] and by Chu & Kimura [17] respectively.

Polyacrylamide-gel electrophoresis was performed with 15% polyacrylamide (15% polyacrylamide and 0.09% bisacrylamide) slab gels (15 cm × 10 cm × 0.1 cm and 15 cm × 15 cm × 0.1 cm) containing 0.1% (w/v) SDS in accordance with Laemmli [18]. The gels were polymerized by addition of 0.1% tetramethylenediamine and 0.1% ammonium persulphate. Electrophoresis was carried out at 170 V/slab gel at room temperature for 2 h. The gels were silver-stained as described by Wray *et al.* [19].

Two-dimensional isoelectric-focusing–SDS/polyacrylamide-gel electrophoresis was performed as described by Andersson & Jörnvall [5].

Incubation procedures

Incubations were carried out for 1–20 min at 37 °C. Vitamin D_3, 1α-hydroxyvitamin D_3, 25-hydroxyvitamin D_3, 5β-cholestane-3α,7α,12α-triol, cholesterol, deoxycorticosterone, lauric acid and testosterone (62.5–250 nmol in 25 μl of acetone) were incubated with 0.05–0.1 nmol of cytochrome *P*-450, 2 nmol of ferredoxin, 0.2 nmol of ferredoxin reductase and 1 μmol of NADPH in a total volume of 1 ml of 50 mM-Tris/acetate buffer, pH 7.4. Incubations with benzphetamine (2 μmol) were performed with 0.1 nmol of cytochrome *P*-450, 2 nmol of ferredoxin, 0.2 nmol of ferredoxin reductase and 2 μmol of NADPH in a total volume of 2 ml of Tris/acetate buffer, pH 7.4. Incubations with vitamin D_3, 25-hydroxyvitamin D_3 and 1α-hydroxyvitamin D_3 were terminated by addition of 5 ml of trichloroethane/methanol (2:1, v/v), incubations with 5β-cholestane-3α,7α,12α-triol, cholesterol, lauric acid and testosterone by addition of 3 ml of 96% (v/v) ethanol, and incubations with deoxycorticosterone by addition of 2 ml of dichloromethane. Incubations with intact mitochondria and vitamin D_3 (500 nmol dissolved in 25 μl of acetone) were performed at 37 °C for 45 min with 4 mg of mitochondrial protein and 15 μmol of isocitrate in a total volume of 10 ml of 50 mM-Tris/acetate buffer, pH 7.4, containing 10 mM-$MgCl_2$ [10,20]. Incubations with intact mitochondria and 5β-cholestane-3α,7α,12α-triol (250 nmol) contained 3 mg of mitochondrial protein, 5 μmol of isocitrate and 30 μmol of $MgCl_2$ in a total volume of 3 ml of 50 mM-Tris/acetate buffer, pH 7.4 [11].

Analysis of incubations

Quantitative determination of 25-hydroxyvitamin D_3 was performed by h.p.l.c. with the use of an LKB 2150 h.p.l.c. pump, LKB 2151 variable-wavelength monitor, Berthold LB 503 D h.p.l.c. radioactivity monitor with a 100 μl flow-through cell (solid-glass scintillator) and a LiChrosorb Si column (150 mm × 3 mm internal diam., 5 μm particle size; Merck) for the straight-phase analysis. A Merck Hitachi 655A-11 liquid chromatograph, Merck Hitachi 655A wavelength u.v. monitor, Merck Hitachi L-5000 liquid-chromatograph controller, Merck Hitachi D-2000 Chromato-Integrator and a LiChrosorb RP-18 column (150 mm × 3 mm internal diam., 5 μm particle size; Merck) were used for the reversed-phase analysis. ^{3}H-labelled 25-hydroxyvitamin D_3 (10000 d.p.m.) was added to the incubations immediately after termination as internal standard to correct for recovery during the extraction and chromatographic procedures. The incubations were extracted twice with trichloroethane/methanol (2:1, v/v). The organic phases were evaporated and the residues were transferred with trichloroethane to vials, dried down with N_2, dissolved in 100 μl of mobile phase and submitted to straight-phase h.p.l.c. analysis. The mobile phase was hexane/propan-2-ol (24:1, v/v) [21] and the solvent flow was 0.7 ml/min. The retention time for 25-hydroxyvitamin D_3 was about 10 min. The fractions corresponding to authentic 25-hydroxyvitamin D_3 were collected, dried down with N_2 and submitted to reversed-phase h.p.l.c. analysis. The residue was dissolved in 100 μl of the mobile phase, aq. 92% (v/v) methanol. The solvent flow was 0.5 ml/min and the retention time for 25-hydroxyvitamin D_3 was 6 min. The amount of 25-hydroxyvitamin D_3 was quantified by comparing the peak found at 265 nm with a standard curve obtained with different amounts of authentic 25-hydroxyvitamin D_3 and corrected for recovery of the added ^{3}H-labelled 25-hydroxyvitamin D_3. Total recovery was about 65% [22].

25-Hydroxyvitamin D_3 was also quantified by isotope dilution–mass spectrometry [4,21], giving essentially the same results as those obtained by h.p.l.c. The quantitative determination of 1α,25-dihydroxyvitamin D_3 formed in incubations with 25-hydroxyvitamin D_3 and 1α-hydroxyvitamin D_3 was performed by isotope dilution–mass spectrometry as described previously [4,23].

Analyses of incubation mixtures with cholesterol and 5β-cholestane-3α,7α,12α-triol with purified cytochrome *P*-450 were performed by t.l.c. as described previously [11]. The developing solvent in the analysis of pregnenolone formation from cholesterol was cyclohexane/ethyl acetate (3:2, v/v). Analyses of incubations with 5β-cholestane-3α,7α,12α-triol were also performed by h.p.l.c. with the same instruments as described above for reversed-phase analysis. N_2-dried incubation extracts were dissolved in 100 μl of mobile phase. The analyses were performed with aqueous solutions and the columns were eluted isocratically. The flow rate was 0.5 ml/min. The mobile phase was aq. 90% (v/v) methanol. The retention time for 5β-cholestane-3α,7α,12α,26-tetrol was 6 min and that for 5β-cholestane-3α,7α,12α-triol 14 min.

The analysis of incubation mixture with testosterone was the same as that described by Boström *et al.* [24]. Incubations with lauric acid were acidified and extracted once with ethyl acetate and once with diethyl ether. The organic phases were combined and washed with water until neutral and subjected to t.l.c. The developing solvent was diethyl ether/n-hexane/acetic acid (1:9:1, by vol.). The chromatoplates were analysed by radioactivity scanning. Incubations of deoxycorticosterone were extracted three times with dichloromethane and analysed by reversed-phase h.p.l.c. The analyses were performed with the same h.p.l.c. instruments as above. The mobile phase was aq. 50% (v/v) methanol. The

Table 1. Purification from livers of untreated rabbits of mitochondrial cytochrome *P*-450 catalysing 25-hydroxylation of vitamin D_3

Details of the purification are given in the Experimental section. Incubations were performed at 37 °C for 5 min except for incubations with intact mitochondria and cholate extract, which were carried out for 45 min. The incubation mixtures contained 0.1 nmol of cytochrome *P*-450, 2 nmol of ferredoxin, 0.2 nmol of ferredoxin reductase, 1 μmol of NADPH and 65 nmol of vitamin D_3 in a total volume of 1 ml of Tris/acetate buffer, pH 7.4. Incubations with intact mitochondria were performed as described in the Experimental section.

	Protein (mg)	Cytochrome *P*-450 (nmol)	Cytochrome *P*-450 (nmol/mg of protein)	Vitamin D_3 25-hydroxylase activity (pmol/min per mg of protein)	Vitamin D_3 25-hydroxylase activity (pmol/min per nmol of cytochrome *P*-450)
Mitochondria	17740	N.D.*	N.D.	0.7	N.D.
Cholate extract	10996	2019	0.18	0.9	5
Octylamine–Sepharose and hydroxyapatite	46.9	132	2.8	154	55
Fractogel TSK DEAE					
Pool 1	9.9	12.9	1.3	< 10	< 10
Pool 2	0.6	4.2	8.5	3570	420
Pool 3	3.4	29.6	8.8	< 10	< 10
Pool 4	1.5	3.9	2.6	< 10	< 10

* Not determined. The cytochrome *P*-450 content in intact mitochondria cannot be accurately measured because of the presence of other cytochromes and cytochrome oxidase. On the basis of the cytochrome *P*-450 content in the cholate extract, which was 0.18 nmol/mg of protein, the content in intact mitochondria can be estimated to be 0.1–0.2 nmol/mg of protein. This is essentially the same cytochrome *P*-450 content as reported for rat liver mitochondria [7].

retention time was 23 min for corticosterone and 49 min for deoxycorticosterone.

Incubations with benzphetamine were analysed for formaldehyde formation by the method of Werringloer [25].

RESULTS AND DISCUSSION

Liver mitochondrial cytochrome *P*-450 catalysing 25-hydroxylation of vitamin D_3 was purified from untreated rabbits. The 25-hydroxylation of vitamin D_3 was analysed at the different steps in the purification procedure. Table 1 summarizes the results of the purification. Chromatography of solubilized mitochondrial cytochrome *P*-450 on octylamine–Sepharose, hydroxyapatite and Fractogel TSK DEAE-650(s) resulted in four pools of cytochrome *P*-450, namely pools 1, 2, 3 and 4. Chromatography on Fractogel TSK DEAE-650(s) instead of DE-52 DEAE-cellulose [11] resulted in more distinct peaks and higher resolution of mitochondrial cytochrome *P*-450. Four peaks, containing cytochrome *P*-450, were obtained in the Fractogel TSK-DEAE-650(s) chromatography, as shown in Fig. 1. The first peak was eluted with the equilibrating buffer, the second and the third in the beginning and the fourth in the middle of the KCl gradient. The fractions with highest cytochrome *P*-450 content from each peak were analysed by SDS/polyacrylamide-gel electrophoresis and pooled to give pools 1, 2, 3 and 4. Cytochrome *P*-450 active in the 25-hydroxylation of vitamin D_3 was present in pool 2. The other fractions (pools 1, 3 and 4) contained cytochrome *P*-450 but showed no or only low activity towards vitamin D_3. The yield of cytochrome *P*-450 in pool 2 represented about 0.2% of the cytochrome *P*-450 present in the cholate extract. The specific cytochrome *P*-450 content increased about 50-fold, from 0.18 nmol/mg of protein in the cholate-solubilized extract to 8.5 nmol/mg of protein in pool 2. The 25-hydroxylase activity, expressed as pmol/min per mg of protein, was about 5000 times higher in the purified cytochrome *P*-450 than in mitochondria. The yield of 25-hydroxylase activity in the isolated cytochrome *P*-450 preparation represented 17% of the activity present in mitochondria.

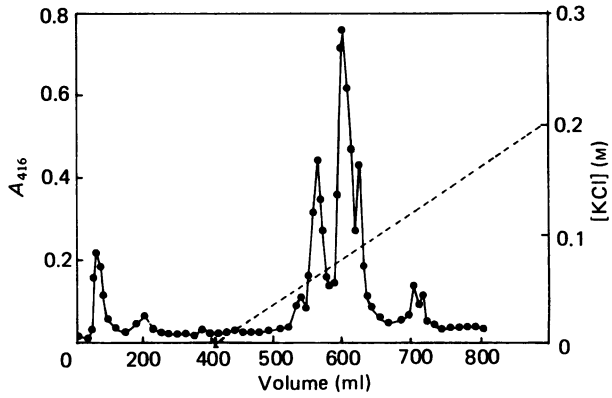

Fig. 1. Fractogel TSK DEAE-650(s) chromatography of mitochondrial cytochrome *P*-450 from livers of untreated rabbits

Cytochrome *P*-450 was solubilized, chromatographed on octylamine–Sepharose and hydroxyapatite (see the Experimental section) and applied to a Fractogel TSK DEAE-650(s) column (1.8 cm × 30 cm) in 10 mM-potassium phosphate buffer, pH 7.4, containing 20% glycerol, 0.05% Emulgen 913 and 0.1% sodium cholate. Cytochrome *P*-450 was eluted as described in the Experimental section. Fractions (3 ml) were collected and analysed for content of cytochrome *P*-450 by measuring the absorbance at 416 nm (●); ----, concentration of KCl.

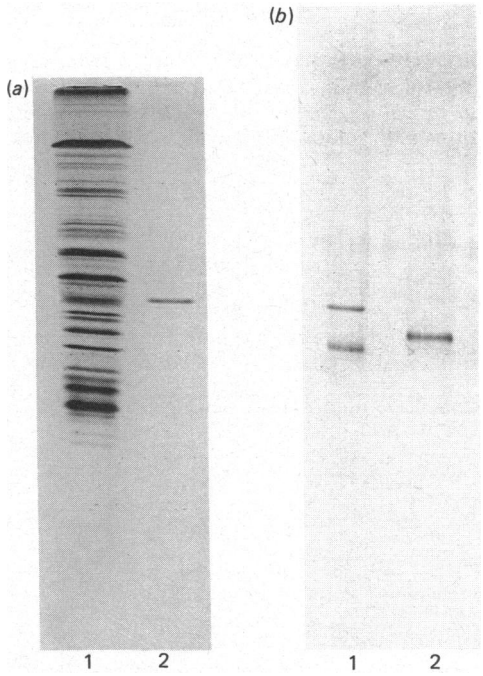

Fig. 2. Polyacrylamide-gel electrophoresis of purified mitochondrial cytochrome *P*-450 from rabbit liver

The protein samples were pretreated with SDS and 2-mercaptoethanol at 100 °C for 2 min and submitted to polyacrylamide-gel electrophoresis in the presence of SDS. Migration was from top to bottom. (*a*) Gel electrophoresis in accordance with Laemmli [18] with modifications as described in the Experimental section. Stacking gels contained 3% (w/v) acrylamide. The separating gels contained 15% (w/v) acrylamide and 0.09% (w/v) bisacrylamide, had a length of 10 cm and were 0.1 cm thick. Lane 1, liver mitochondria, 40 μg; lane 2, mitochondrial cytochrome *P*-450, 0.5 μg. (*b*) The same gel system as in (*a*) but with separating gels having a length of 15 cm. Lane 1, mixture of cytochrome *P*-450 LM_4 (M_r 56000) and cytochrome *P*-450 LM_2 (M_r 50000) from rabbit liver microsomal fraction, 0.5 μg of each; lane 2, mitochondrial cytochrome *P*-450, 0.5 μg.

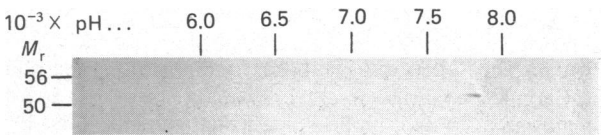

Fig. 3. Two-dimensional isoelectric-focusing–polyacrylamide-gel electrophoresis of mitochondrial cytochrome *P*-450 from rabbit liver

A sample containing 2 μg of mitochondrial cytochrome *P*-450 was subjected to two-dimensional isoelectric-focusing–polyacrylamide-gel electrophoresis as described in the Experimental section. Indicated are the electrophoretic migrations of proteins of known M_r, namely cytochrome *P*-450 LM_4 (M_r 56000) and cytochrome *P*-450 LM_2 (M_r 50000) from rabbit liver microsomal fraction. The pH gradient in the tube gel was determined by subjecting a parallel gel to isoelectric focusing, after which the gel was cut into consecutive 5 mm slices that were incubated in degassed water overnight at 4 °C before pH measurement.

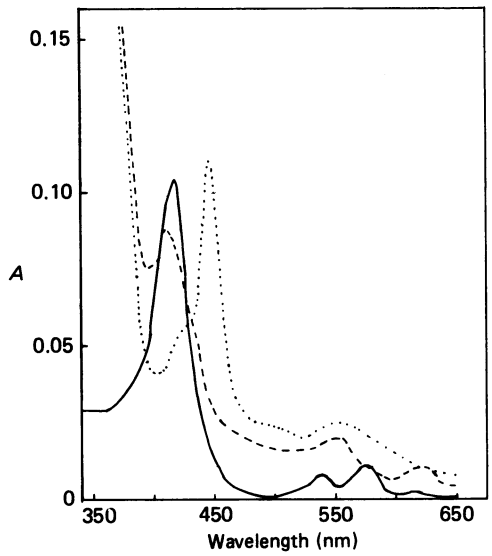

Fig. 4. Absolute spectra of purified mitochondrial cytochrome *P*-450 from livers of untreated rabbits

The concentration of the cytochrome *P*-450 was 0.56 nmol/ml of 50 mM-potassium phosphate buffer, pH 7.4, containing 20% glycerol. ——, Oxidized cytochrome *P*-450; – – – –, reduced cytochrome *P*-450; · · · · · ·, reduced cytochrome *P*-450–CO complex.

Fig. 2 shows the results of SDS/polyacrylamide-gel electrophoresis of the 25-hydroxylating cytochrome *P*-450. The preparation showed only one protein band, corresponding to an apparent M_r of 52000. The M_r of the mitochondrial cytochrome *P*-450 differed from that of the microsomal cytochromes *P*-450 LM_2 and LM_4. To examine the purity of the preparation further, experiments were performed in which the cytochrome *P*-450 preparation was submitted to two-dimensional isoelectric-focusing–polyacrylamide-gel electrophoresis. A single spot with an apparent isoelectric point of 7.8 and an M_r of about 52000 was observed (Fig. 3). The spectral properties of the mitochondrial cytochrome *P*-450 are shown in Fig. 4. The fraction showed an absorbance maximum in the oxidized state at 418 nm and in the reduced state at 410 nm. The absorbance maximum for the reduced CO complex was at 447 nm. The spectral properties indicate a low-spin form of cytochrome *P*-450 [26].

The reconstitution conditions for the 25-hydroxylation of vitamin D_3 were analysed in a series of experiments, and the results are summarized in Table 2. The activity showed an absolute requirement for the cytochrome *P*-450, ferredoxin and ferredoxin reductase as well as for NADPH. Ferredoxin and ferredoxin reductase could not be replaced by microsomal NADPH–cytochrome *P*-450 reductase from rabbit liver. Fig. 5 shows that the conversion of vitamin D_3 into 25-hydroxyvitamin D_3 was approximately linear with the amount of cytochrome *P*-450 up to about 0.2 nmol and with time up to 10 min. The system was saturated with 2 nmol of ferredoxin and 0.2 nmol of ferredoxin reductase and with a 65 μM concentration of the substrate. The apparent K_m for the 25-hydroxylation of vitamin D_3 in the reconstituted system was measured in separate experiments and was found to be 10 μM. This value is about the same as that

Table 2. Reconstitution conditions for 25-hydroxylation of vitamin D_3 by the purified mitochondrial cytochrome *P*-450

Incubations were performed as described in Table 1 except for the omission of cytochrome *P*-450, NADPH, ferredoxin and/or ferredoxin reductase in some experiments. In the experiment with microsomal NADPH–cytochrome *P*-450 reductase, 1.5 units of this protein were used.

Components	25-Hydroxylation (pmol/min per nmol of cytochrome *P*-450)
Cytochrome *P*-450	< 10
Ferredoxin + ferredoxin reductase	< 10
Cytochrome *P*-450 + ferredoxin	< 10
Cytochrome *P*-450 + ferredoxin reductase	< 10
Cytochrome *P*-450 + ferredoxin + ferredoxin reductase*	340
Cytochrome *P*-450 + microsomal NADPH–cytochrome *P*-450 reductase	< 10

* Omission of NADPH from the reconstituted system resulted in no detectable 25-hydroxylase activity.

reported in previous studies with purified cytochrome *P*-450 from liver microsomal fraction [6,27] and with partially purified cytochrome *P*-450 from liver mitochondria [8]. It might be mentioned that the concentration of vitamin D_3 in the whole liver has been estimated to be around 0.1 μM [28]. No information is available concerning the concentration of vitamin D_3 in the different subcellular components of liver.

The catalytic properties of the 25-hydroxylating mitochondrial cytochrome *P*-450 are summarized in Table 3. The preparation catalysed the 25-hydroxylations of vitamin D_3 and 1α-hydroxyvitamin D_3. In addition, it catalysed the 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol. The rate of 26-hydroxylation of this C_{27} steroid was 20 and 50 times higher respectively than the rate of 25-hydroxylation of 1α-hydroxyvitamin D_3 and vitamin D_3. The cytochrome *P*-450 was inactive in side-chain cleavage of cholesterol and in 11β-hydroxylation of deoxycorticosterone, reactions catalysed by cytochromes *P*-450 from adrenal mitochondria [29]. The lack of 1α-hydroxylase activity towards 25-hydroxyvitamin D_3 shows that it is catalytically distinct also from cytochrome(s) *P*-450 in kidney mitochondria [30]. The enzyme was inactive in hydroxylations of lauric acid and testosterone and in demethylation of benzphetamine, reactions that are carried out by many cytochromes *P*-450 isolated from liver microsomal fractions [29,31–33].

The finding that the 25-hydroxylating cytochrome *P*-450 also catalysed an efficient 26-hydroxylation of 5β-cholestane-3α,7α,12α-triol prompted experiments in which the 26-hydroxylase activity was analysed at different steps in the purification. The ratio between the 26-hydroxylase and the 25-hydroxylase activities was about 60:1 in the mitochondria and remained constant throughout the purification procedure. Pool 1 from the Fractogel TSK DEAE-650(s) chromatography did not

Table 3. Hydroxylase activities in purified liver mitochondrial cytochrome *P*-450 from untreated rabbits

The incubations were performed at 37 °C for 5 min with vitamin D_3, 3 min with 5β-cholestane-3α,7α,12α-triol and 20 min with the other substrates. The reaction mixtures contained 0.05 nmol of cytochrome *P*-450 in the incubation with 5β-cholestane-3α,7α,12α-triol and 0.1 nmol of cytochrome *P*-450 in the incubations with the other substrates, 2 nmol of ferredoxin, 0.2 nmol of ferredoxin reductase and 1 μmol of NADPH in a total volume of 1 ml of 50 mM-Tris/acetate buffer, pH 7.4. The following amounts of substrates were used; vitamin D_3, 65 nmol; 1α-hydroxyvitamin D_3 and 25-hydroxyvitamin D_3, 160 nmol; 5β-cholestane-3α,7α,12α-triol, 125 nmol; cholesterol, 25 nmol; deoxycorticosterone, 30 nmol; lauric acid, 250 nmol; testosterone, 175 nmol; benzphetamine, 2 μmol.

Reaction	Cytochrome *P*-450 (pmol/min per nmol of cytochrome *P*-450)
Vitamin D_3 25-hydroxylation	395
1α-Hydroxyvitamin D_3 25-hydroxylation	1200
25-Hydroxyvitamin D_3 1α-hydroxylation	< 10
5β-Cholestane-3α,7α,12α-triol 26-hydroxylation	25000
Cholesterol side-chain cleavage	< 10
Deoxycorticosterone 11β-hydroxylation	< 10
Lauric acid $\omega+(\omega-1)$-hydroxylation	< 10
Testosterone hydroxylations	< 10
Benzphetamine demethylation	< 1000

contain any detectable 26-hydroxylase activity. Pools 3 and 4 catalysed 26-hydroxylation, but at a considerably lower rate than that catalysed by the 25-hydroxylating cytochrome *P*-450 in pool 2 (cf. Fig. 1).

The results of the present study show that the properties of the mitochondrial cytochrome *P*-450 active in the 25-hydroxylation of vitamin D_3 are similar to those of a mitochondrial cytochrome *P*-450, active in 26-hydroxylation of C_{27} steroids, isolated earlier from rabbit liver mitochondria [11]. Thus the 26-hydroxylase activity, the chromatographic behaviour, the haem content and the spectral properties are essentially the same for the two cytochrome *P*-450 preparations. In a separate set of experiments (results not shown) it was found that the two preparations also show the same apparent M_r in the gel-electrophoretic system used in the present work. These findings raise the question whether the 25-hydroxylation of vitamin D_3 and the 26-hydroxylation of C_{27} steroids are catalysed by a common cytochrome *P*-450 species in liver mitochondria.

There are several lines of evidence indicating that mitochondrial cytochrome *P*-450 plays an important role in the metabolic activation of vitamin D_3 in the liver. According to DeLuca [30] the microsomal 25-hydroxylation of vitamin D_3 is more important than the mitochondrial 25-hydroxylation in the bioactivation process under normal conditions. This contention is based on experiments mainly with male rats. However,

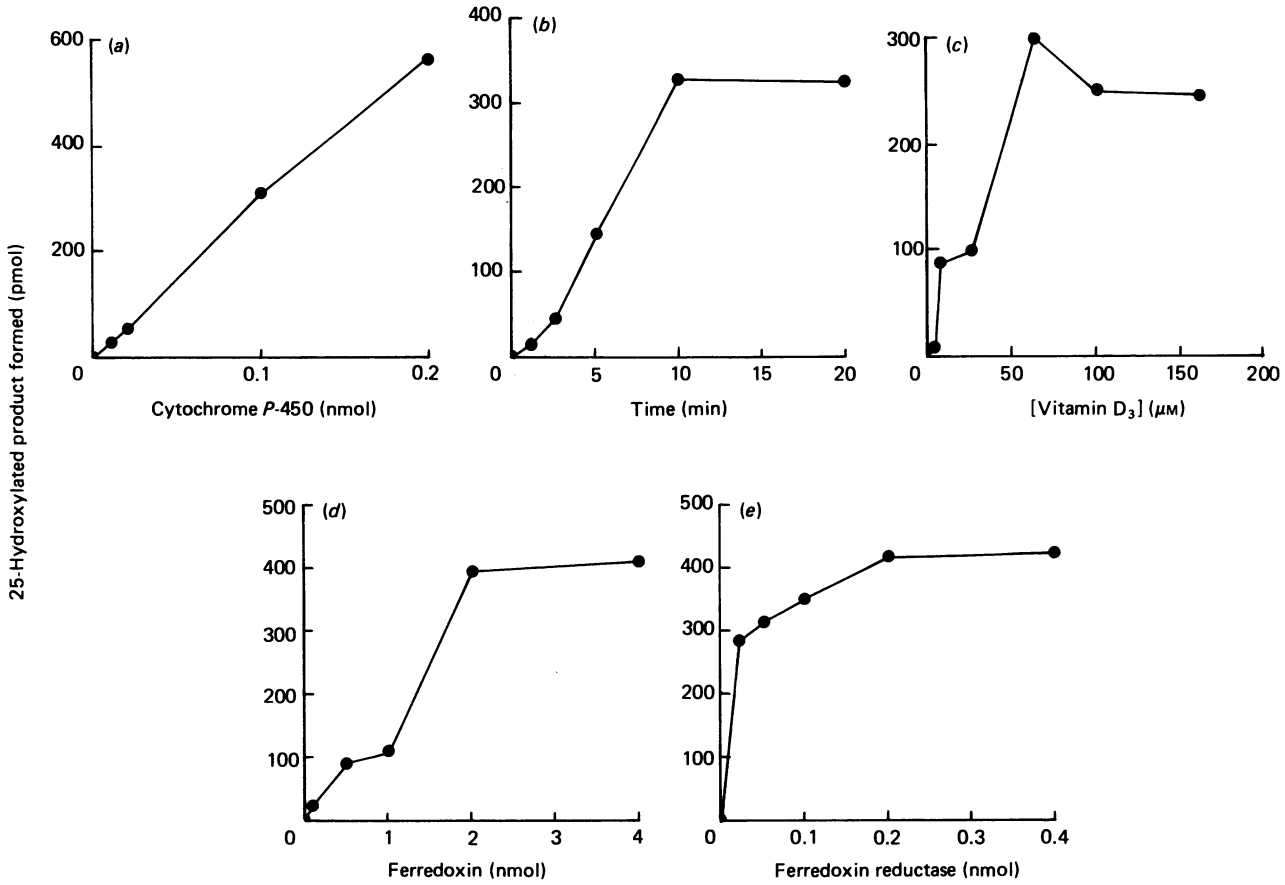

Fig. 5. Effect of cytochrome *P*-450 concentration (*a*), time (*b*), vitamin D_3 concentration (*c*), ferredoxin (*d*) and ferredoxin reductase (*e*) concentrations on the rate of 25-hydroxylation of vitamin D_3

Incubations were performed as described in Table 1 except when the concentration of a component was varied.

the role of the microsomal fraction in 25-hydroxylation has been recently questioned. The present mitochondrial cytochrome *P*-450 from rabbit liver has a turnover for 25-hydroxylation of vitamin D_3 that is more than 100 times higher than that reported earlier for a microsomal cytochrome *P*-450 from rabbit liver [27]. Further, Pedersen and collaborators have reported that 25-hydroxylation of vitamin D_3 [22] as well as of 1α-hydroxyvitamin D_3 [34] is catalysed only by the mitochondrial fraction of human liver. It might be mentioned, however, that Holmberg *et al.* [35] recently showed that partially purified cytochrome *P*-450 from human liver microsomal fraction was not completely devoid of 25-hydroxylase activity. Andersson *et al.* [4] have purified to homogeneity a cytochrome *P*-450 from the liver microsomal fraction of male rats that is active in 25-hydroxylation of vitamin D_3. Experiments with a monospecific monoclonal antibody showed that this microsomal 25-hydroxylating cytochrome *P*-450 was male-specific and not present in the liver microsomal fraction of female rats [5]. On the other hand, mitochondrial cytochrome *P*-450 from livers of female rats catalyses the 25-hydroxylation of vitamin D_3 as efficiently as the same material from male rats [10]. Taken together, these results suggest that the liver mitochondrial cytochrome *P*-450 isolated in the present study plays an important role in the metabolism of vitamin D_3.

The skilful technical assistance of Mrs. Kerstin Rönnqvist is gratefully acknowledged. We are grateful to Professor Ingemar Björkhem for placing mass-spectrometric equipment at our disposal and to Dr. Inger Holmberg for valuable advice in connection with the mass-fragmentographic assay of 25-hydroxyvitamin D_3 and 1α,25-dihydroxyvitamin D_3. This work was supported by the Swedish Medical Research Council (Project 03X-218).

REFERENCES

1. Bhattacharayya, M. H. & DeLuca, H. F. (1974) Arch. Biochem. Biophys. **160**, 58–62
2. Madhok, T. C. & DeLuca, H. F. (1979) Biochem. J. **184**, 491–499
3. Björkhem, I. & Holmberg, I. (1978) J. Biol. Chem. **253**, 842–849
4. Andersson, S., Holmberg, I. & Wikvall, K. (1983) J. Biol. Chem. **258**, 6777–6781
5. Andersson, S. & Jörnvall, H. (1986) J. Biol. Chem. **261**, 16932–16936
6. Hayashi, S., Noshiro, M. & Okuda, K. (1986) J. Biochem. (Tokyo) **99**, 1753–1763
7. Pedersen, J. I., Holmberg, I. & Björkhem, I. (1979) FEBS Lett. **98**, 394–398
8. Björkhem, I., Holmberg, I., Oftebro, H. & Pedersen, J. I. (1980) J. Biol. Chem. **255**, 5244–5249
9. Oftebro, H., Saarem, K., Björkhem, I. & Pedersen, J. I. (1981) J. Lipid Res. **22**, 1254–1264

10. Dahlbäck, H. & Wikvall, K. (1987) Biochem. Biophys. Res. Commun. **142**, 999–1005
11. Wikvall, K. (1984) J. Biol. Chem. **259**, 3800–3804
12. Cronholm, T. & Johansson, G. (1970) Eur. J. Biochem. **16**, 373–381
13. Cuatrecasas, P. (1970) J. Biol. Chem. **245**, 3059–3065
14. Omura, T. & Sato, R. (1964) J. Biol. Chem. **239**, 2379–2385
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
16. Huang, J. J. & Kimura, T. (1973) Biochemistry **12**, 406–409
17. Chu, J.-W. & Kimura, T. (1973) J. Biol. Chem. **248**, 2089–2094
18. Laemmli, U. K. (1970) Nature (London) **227**, 680–685
19. Wray, W., Boulikas, T., Wray, V. & Hancock, R. (1981) Anal. Biochem. **118**, 197–203
20. Björkhem, I. & Holmberg, I. (1979) J. Biol. Chem. **254**, 9518–9524
21. Holmberg, I., Kristiansen, T. & Sturén, M. (1984) Scand. J. Clin. Lab. Invest. **44**, 275–282
22. Saarem, K., Bergseth, S., Oftebro, H. & Pedersen, J. I. (1984) J. Biol. Chem. **259**, 10936–10940
23. Björkhem, I., Holmberg, I., Kristiansen, T. & Pedersen, J. I. (1979) Clin. Chem. **25**, 584–588
24. Boström, H., Hansson, R., Jönsson, K.-H. & Wikvall, K. (1981) Eur. J. Biochem. **120**, 29–32
25. Werringloer, J. (1978) Methods Enzymol. **52C**, 297–302
26. Haugen, D. A. & Coon, M. J. (1976) J. Biol. Chem. **251**, 7929–7939
27. Hansson, R., Holmberg, I. & Wikvall, K. (1981) J. Biol. Chem. **256**, 4345–4349
28. Holmes, R. P. & Kummerow, F. A. (1983) J. Am. Coll. Nutr. **2**, 173–199
29. Coon, M. J. & Koop, D. R. (1983) Enzymes 3rd Ed. **16**, 645–677
30. DeLuca, H. F. (1980) Harvey Lect. **75**, 333–379
31. Lu, A. Y. H., Junk, K. W. & Coon, M. J. (1969) J. Biol. Chem. **244**, 3714–3721
32. Gibson, G. G., Orton, T. C. & Tamburini, P. P. (1982) Biochem. J. **203**, 161–182
33. Ryan, D. E., Thomas, P. E., Korzeniowski, D. & Levin, W. (1979) J. Biol. Chem. **254**, 1365–1374
34. Saarem, K. & Pedersen, J. I. (1985) Biochim. Biophys. Acta **840**, 117–126
35. Holmberg, I., Berlin, T., Ewerth, S. & Björkhem, I. (1986) Scand. J. Clin. Lab. Invest. **46**, 785–790

Received 3 August 1987/6 November 1987; accepted 21 January 1988